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miR-146a and NF-κB1 Regulate Mast Cell Survival and T Lymphocyte Differentiation

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The transcription factor NF-κB regulates the expression of a broad number of genes central to immune and inflammatory responses. We identified a new molecular network that comprises specifically the NF-κB family member NF-κB1 (p50) and miR-146a, and we show that in mast cells it contributes to the regulation of cell homeostasis and survival, while in T lymphocytes it modulates T cell memory formation. Increased mast cell survival was due to unbalanced expression of pro- and antiapoptotic factors and particularly to the complete inability of p50-deleted mast cells to induce expression of miR-146a, which in the context of mast cell survival acted as a proapoptotic factor. Interestingly, in a different cellular context, namely, human and mouse primary T lymphocytes, miR-146a and NF-κB p50 did not influence cell survival or cytokine production but rather T cell expansion and activation in response to T cell receptor (TCR) engagement. Our data identify a new molecular network important in modulating adaptive and innate immune responses and show how the same activation-induced microRNA (miRNA) can be similarly regulated in different cell types even in response to different stimuli but can still determine very different outcomes, likely depending on the specific transcriptome.

The NF-κB family of transcription factors comprises five related proteins (c-Rel, RelA, RelB, NF-κB1 [p50], and NF-κB2), which are critical regulators of immunity, stress response, apoptosis, and differentiation and bind as dimers to kB sites in promoters and enhancers of a variety of genes to induce or repress transcription (reviewed in reference 25). The crucial role played by this transcription factor in orchestrating immune responses is highlighted by the number of stimuli that can elicit NF-κB activation, including bacterial and viral infections, inflammatory cytokines, and engagement of antigen (Ag) receptors. As a consequence, dysregulation of NF-κB activity is linked to inflammatory disorders and autoimmune diseases, as well as cancer (25). Given the wide range of cellular responses regulated by NF-κB, it is not surprising that its activity must be tightly controlled at multiple levels by positive and negative regulatory elements. MicroRNAs (miRNAs) are now widely recognized as modulators of many aspects of immune responses (13). miR-146a in particular is a well-studied modulator of the immune system (31), known to regulate NF-κB activation and tolerance in innate immunity (36), to act as an oncosuppressor, and to modulate T regulatory (Treg) cell functions (17, 42).

Mast cells are key effector cells in immediate hypersensitivity reactions and allergic disorders. Mice lacking the transcription factor p50 (p50ko) show impaired airway eosinophilic inflammation in the lung due to the inability to produce interleukin-4 (IL-4), IL-5, and IL-13 and to a defect in the polarization of Th2 lymphocytes (5, 29, 41). Despite the important role of mast cells in allergy and asthma and as a source of Th2-type cytokines, mast cell responses were never specifically evaluated in these mice. Here, we investigated whether p50 may have a role in regulating mast cell differentiation, homeostasis, and function, as it could improve our understanding of the molecular mechanisms causing mast cell-related diseases such as asthma, allergy, and even mastocytosis. Specifically, we identified a role for p50, but also for miR-146a, whose transcription was completely dependent on p50, in regulating mast cell homeostasis and cell survival. Interestingly, the same molecular network involving p50 and miR-146a acted also at the level of T lymphocytes to modulate immunological memory. Memory T cells can be broadly separated into central memory (T_{CM}) cells that express the chemokine receptor CCR7 and recirculate through lymphoid organs and effector memory (T_{EM}) cells that lack CCR7 and preferentially home to nonlymphoid tissues (33). Specifically, we found that the absence of p50 (and as a consequence, of miR-146a) led preferentially to a T_{CM} phenotype and, accordingly, that both human and mouse T cells were forced to express higher levels of miR-146a preferentially differentiated toward a T_{EM}-like phenotype.

Overall, we provide evidence that in the absence of p50, mast cells showed altered tissue homeostasis and survival due to increased expression of prosurvival factors such as Bcl-2 and A1, as well as reduced expression of proapoptotic factors such as Bax and miR-146a. The latter in particular acted in this context as a modulator of NF-κB signaling by targeting TRAF6 and reducing mast cell survival. Interestingly, in T cells miR-146a had no role in regulating T cell survival or cytokine production, but it emerged as an important regulator of T cell expansion and memory formation.

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MATERIALS AND METHODS

Cell cultures and cell stimulation. Bone marrow-derived mast cells (BMMCs) from C57BL/6 mice and p50-deleted mice (34) were differentiated *in vitro* by culturing total bone marrow cells for at least 3 weeks in Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 50 μM β-mercaptoethanol, antibiotics, and 50% WEHI-3 conditioned supernatant as a source of IL-3. WEHI-3 conditioned supernatant was prepared exactly as described previously (19). When indicated, stem cell factor (SCF) (Peprotech) (10 ng/ml) was also added to the cultures during mast cell differentiation. Cell differentiation was assessed by surface staining for FcεRI and Kit receptor and by toluidine blue staining as described previously (19). Mast cells were acutely stimulated either with 1.5 μg/ml IgE-antidinitrophenyl (DNP) (clone SPE7; Sigma) and 0.2 μg/ml DNP-human serum albumin (DNP-HSA) (Sigma) or with 20 to 100 μg/ml lipopolysaccharide (LPS), depending on the experimental conditions. In some cases, cells were stimulated with 20 nM phorbol myristate acetate (PMA) and 2 μM ionomycin. Primary human T lymphocytes were purified from peripheral blood and expanded as described previously (21). T cells were stimulated with immobilized anti-CD3 (1 to 10 μg/ml) and anti-CD28 (2 μg/ml) and expanded in the presence of 500 U/ml recombinant human IL-2 (rhIL-2). Blood from healthy donors was obtained from the Swiss Blood Donation Center of Basel and Lugano and used in compliance with the Federal Office of Public Health. Mouse naïve CD8 and CD4 T cells were purified using beads (Dynal or Miltenyi Biotec) from spleen and lymph nodes of OT-I, OT-II, or C57BL/6 mice and identified by fluorescence-activated cell sorter (FACS) analysis as CD62L positive ($CD62L^+$) and $CD44^-$. Naïve CD4 T cells were cultured under Th1/Th2 skewing conditions exactly as described previously (1, 22). All animal studies were performed in accordance with the Swiss Federal Veterinary Office guidelines and were approved by the Dipartimento della Sanità e della Socialità (approval no. 17/2010, 18/2010, and 03/2012).

Plasmids and lentiviral and retroviral transductions. The control lentiviral vectors were previously described and expressed a small hairpin RNA (shRNA) against luciferase (shLuc), a nontargeting hairpin (NT), or green fluorescent protein (GFP) alone (18). The 394-bp PCR fragment encompassing the pre-miR-146a genomic sequence was cloned using standard cloning techniques. Depending on the experimental conditions, transduced cells were either selected with puromycin (2 μg/ml for 2 days) or FACS sorted for GFP expression. To optimize transgene expression, the same miR-146a or control insert was driven by the spleen focus-forming virus promoter (SFFVp) for transductions of mast cells and by the EF1 alpha promoter for transductions of human T lymphocytes. Lentiviral particles were produced exactly as previously described and used at a multiplicity of infection of ~60 (19, 44). Retroviral particles were generated by transient transfection of Phoenix cells as previously described (24).

qRT-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen). To analyze miRNA expression, quantitative reverse transcription-PCR (qRT-PCR) was performed using a miRNA reverse transcription kit and TaqMan miRNA assays from Applied Biosystems, exactly following the manufacturer's instructions. To analyze Bcl-2 family member expression, total RNA (1 μg) was reverse transcribed using an iScript kit (Bio-Rad) before PCR amplification was performed with the following primers: *bcl2* FW (5′-TTCGCAGCGATGTCCAGTCAGCT) and *bcl2* RV (5′-TGAAG AGTTCTTCCACCACCGT); *A1* FW (5′-GATTGCCCTGGATGTATGT GCTTA) and *A1* RV (5′-AGCCATCTTCCCAACCTCCATTC); *bcl-X_L* FW (5′-CAGTGCCATCAATGGCAACCCATC) and *bcl-X_L* RV (5′-CGC AGTTCAAACTCATCGCCTGC); and *bax* FW (5′-ACTGGACAGCAAT ATGGAGCTG) and *bax* RV (5′-CCCAGTTGAAGTTGCCATCAG). β-*actin* was used as an endogenous control.

Apoptosis and proliferation. For cell death analysis, mast cells were washed extensively to remove all IL-3 and/or SCF from the culture medium and either were left resting or were stimulated with IgE and antigen. Apoptosis was evaluated using an annexin V-phycoerythrin (PE) apoptosis kit (BD Pharmingen) following the manufacturer's instructions. For thymidine incorporation assays, 1×10^5 mast cells were stimulated for 24 h, and in the last 16 h of incubation, 1 μCi/ml of [^{3}H]thymidine (GE Healthcare) was added to the cultures. Cells were then collected, and levels of thymidine incorporation were evaluated with a scintillation beta counter.

Degranulation assay. Mast cell degranulation was assessed as described previously (18). Briefly, 5×10^4 cells were resuspended in 50 μl Opti-MEM–1% FBS and stimulated for 1 h with either PMA and ionomycin or IgE-antigen complexes. The supernatant was collected, and the cell pellet was lysed in 50 μl of 0.5% Triton X-100–Opti-MEM–1% FBS. The β-hexosaminidase substrate (4-nitrophenyl *N*-acetyl-β-D-glucosaminidate [Sigma]) was then added to both the cell lysates and supernatants (50 μl of a 3.8 mM solution). After incubation for 2 h at 37°C, the reaction was stopped with 90 μl glycine (0.2 M; pH 10.7), and the absorbance was read at 405 nm. The percentage of degranulation was calculated as the ratio between the absorbance of supernatants and the total absorbance of supernatants and cell lysates.

Intracellular cytokine staining. Cells were stimulated with PMA and ionomycin, IgE and antigen complexes, or LPS for 3 to 5 h, with addition of 10 μg/ml brefeldin-A in the last 2 h of stimulation. The cells were then fixed with 4% paraformaldehyde and permeabilized in 0.5% saponin–1% bovine serum albumin (BSA) prior staining with fluorescent anticytokine antibodies (eBioscience) and FACS analysis.

Western blot analysis and immunofluorescence staining. For Western blot analyses, total protein extracts were prepared by direct lysis of the cells in Laemmli sample buffer. Samples were separated on 12% SDS-polyacrylamide gels, and immunodetection was performed with antibodies against NF-κB p50 (NLS), tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) (H-274), and, as a loading control, β-tubulin (H-235) (all from Santa Cruz Biotechnologies). Quantification was performed with a biomolecular imager (ImageQuant LAS 4000). For immunofluorescence staining, tissue slides of paraffin-embedded organs from wild-type and p50-deleted mice were deparaffinized, rehydrated, and stained with 2 μg anti-mast cell tryptase antibody (FL-275; Santa Cruz Biotechnologies), followed by an anti-rabbit Alexa Fluor 594-conjugated secondary antibody (Invitrogen). Nuclei were counterstained with 4′,6′diamidino-2-phenylindole (DAPI). Images were captured with a Nixon Eclipse E800 microscope and analyzed with Openlab software (Improvision).

PCA and peritoneal lavages. For passive cutaneous anaphylaxis (PCA) experiments, 1×10^6 differentiated BMMCs were injected intradermally (i.d.) in the ear pinna of mice lacking mast cells ($Kit^{W\text{-}sh/W\text{-}sh}$; Jackson Laboratory) (8). Four weeks after reconstitution, transferred mast cells were sensitized by i.d. injections of IgE–anti-DNP (1.5 μg/ml) and challenged 24 h later with intravenous (i.v.) injections of 250 μg/ml DNP-HSA together with 5 mg/ml Evans blue dye to assess extravasation. Mice were sacrificed 30 min after challenge, and the blue dye was extracted from the tissues by incubation in formamide at 63°C overnight (O/N). The intensity of the blue dye (correlating with the extent of extravasation and therefore mast cell activation) was measured spectrophotometrically (optical density at 600 nm [OD_{600}]). The presence of mast cells in the reconstituted ears was assessed by toluidine-blue staining of paraffin-embedded ears. For peritoneal lavages, the total number of recovered cells was assessed by manual counting, while the percentage of mast cells was evaluated by surface staining for Kit and FcεRIα and FACS analysis.

Transfection of naïve T cells. Sorted naïve ($CD4^+$ $CD8^-$ $CD62L^{hi}$ $CD44^{lo}$) T cells were transfected with an Amaxa mouse T cell nucleofector kit following the manufacturer's instructions and using program X-01. Cells were rested for at least 3 h after transfection and prior stimulation with plate-bound anti-CD3 and anti-CD28 for 48 h.

Adoptive transfer of mouse T cells and immunization. Sorted CD8- or CD4-naïve T cells were obtained from spleen and lymph nodes of C57BL/6, OT-I, or OT-II mice and injected (1×10^6 cells/mouse) into C57BL/6 recipients. Mice were then challenged subcutaneously with com-

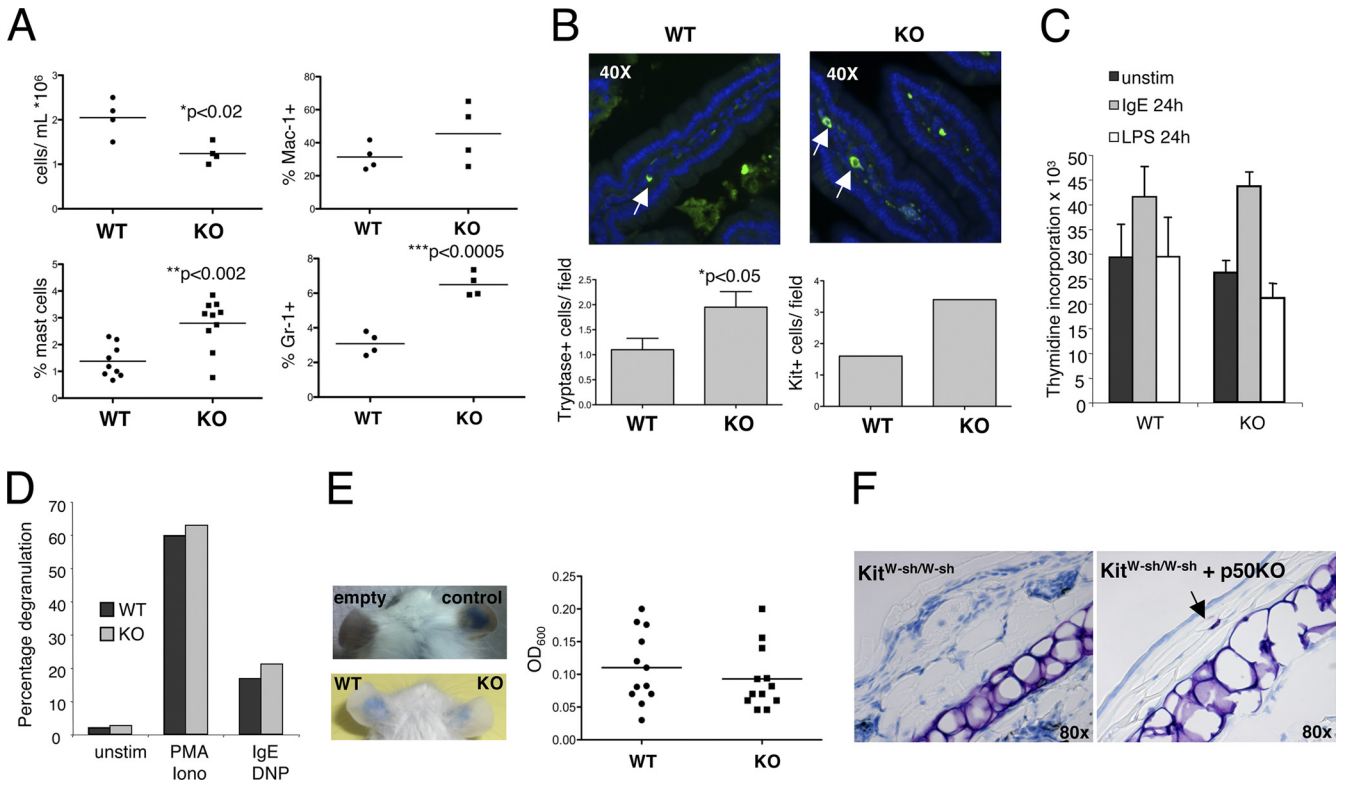

FIG 1 Increased tissue mast cells in mice lacking p50. (A) Peritoneal lavage was performed on control and p50ko mice, and total cell numbers and percentages of mast cells (Kit^+ $Fc\varepsilon RI^+$) and Mac-1^+ and Gr-1^+ cells were assessed by manual counting and FACS staining, respectively. Each dot represents one animal. WT, wild type; KO, knockout. (B) Immunofluorescence staining of small-intestine sections of control and p50ko mice. Mast cells were identified by staining with an antitryptase antibody (top images and left graph) or an anti-Kit antibody (right graph). At least 10 fields per tissue slide were observed and counted in a blinded manner (the operator counting the cells was not aware of the type of sample, which was coded), and the observed percentages of $tryptase^+$ and Kit^+ cells are plotted (bottom). (C) Mast cell proliferation was assessed by thymidine incorporation assays. Control and p50ko mast cells either were left resting (unstim) or were stimulated for 24 h with IgE-Ag complexes or LPS prior addition of ^{3}H-thymidine. Data represent the results of one of three experiments. (D) To assess mast cell degranulation in response to acute stimulation *in vitro*, mast cells either were left untreated or were stimulated with IgE-Ag complexes or LPS for 1 h prior measurement of β-*N*-acetylhexosaminidase release in degranulation assays. Data represent the results of one of three experiments. Iono, ionomycin. (E) Mast cells from C57BL/6 and p50ko mice were differentiated and injected intradermally (i.d.) into the ear pinna of $Kit^{W\text{-}sh/W\text{-}sh}$ mice. Four weeks after injection, mast cells were sensitized by i.d. injection of IgE–anti-DNP and then intravenously challenged 24 h later with DNP-HSA and Evans blue to assess extravasation. The intensity of blue staining in ear tissue was analyzed spectrophotometrically (OD_{600}) after extraction with formamide. Pictures on the left show the results of one representative experiment, and the graph shows the quantification of several experiments. Each dot represents one mouse. (F) Effective mast cell reconstitution of the ear pinna performed as described for panel E was assessed by fixation and embedding in paraffin of the ear tissue followed by toluidine blue staining.

plete Freund's adjuvant-ovalbumin (CFA-OVA), and naïve, effector, and memory cells were obtained from the spleen and draining lymph nodes 4 days (effector) or 2 weeks (memory) after challenge, and they were sorted into different subsets based on the expression of CD4, CD8, CD62L, CD44, and CD127.

CFSE labeling. Carboxyfluorescein succinimidyl ester (CFSE) labeling was performed using a CellTrace cell proliferation kit (Invitrogen). Briefly, 1×10^6 to 1×10^7 naïve T cells (human or mouse) were labeled with 5 μM CFSE for 8 min at 37°C prior extensive washing and antigenic stimulation.

Statistical analysis. Results are expressed as means ± standard deviations (SD) or standard errors of the means (SEM). Comparisons were made using the unpaired *t* test and the GraphPad Prism software.

RESULTS

Elevated numbers of mast cells in the absence of NF-κB p50. Mice lacking p50 (p50ko) show impaired airway eosinophilia inflammation due to the inability to produce IL-4, IL-5, and IL-13 and to a defect in the polarization of Th2 lymphocytes (5, 29, 41). Since mast cells are master effector cells in asthmatic and allergic responses, we evaluated whether such an asthma-resistant phenotype could be partially due to a defect in mast cell development or function. First, we evaluated the numbers of mast cells in the tissues of p50ko animals. In the peritoneal lavage fluids of control and p50ko mice, the total number of cells and the percentage of mast cells were assessed by manual counting and by surface staining for FcεRIα and Kit, respectively. Surprisingly, the percentage of mast cells recovered from the peritoneal cavity of p50ko mice was significantly augmented ($P < 0.002$) compared to control animals (Fig. 1A). The percentage of Gr-1^+ cells was also significantly increased in the peritoneal lavage fluid of p50ko mice relative to controls. A similar increase in the presence of mast cells was also observed in the small intestine of p50ko animals compared to C57BL/6 mice, as assessed by immunofluorescence staining using either of two different mast cell markers (tryptase or Kit) (Fig. 1B). These results showed an overall increase in mast cell numbers in the tissues of p50ko animals without, however, revealing any alteration in the homing properties of these cells. Indeed, mast cells were found in all tissues and compartments where they are normally present but not in organs, such as the spleen or liver, where

they are usually not found (not shown). Having ruled out the possibility that the asthma-resistant phenotype of p50ko animals does not correlate with the number of mast cells in these mice, and considering that mast cells are able to produce very large amounts of (especially) IL-13, we assessed the ability of these cells to respond to a variety of stimuli.

Reduced cytokine production in mast cells lacking p50. Similarly to the cells of the controls, *in vitro*-differentiated, bone marrow-derived p50ko mast cells were homogenously FcεRIα$^+$ Kit$^+$, expressed granzyme B and mouse mast cell protease 5 (mMCP5) (not shown), and looked phenotypically normal, as assessed by toluidine blue staining (see Fig. S1A in the supplemental material). Since the percentage of mast cells was increased in the tissues of p50ko animals relative to C57BL/6 mice, we asked whether the absence of p50 could favor cell proliferation. As assessed by a thymidine incorporation assay, p50ko cells proliferated similarly to the cells of the controls under all conditions tested (Fig. 1C). Next, we evaluated whether the impaired Th2 responses observed in these mice could be partially explained by an altered ability of mast cells to perform their effector functions (degranulation, cytokine production) in response to acute stimulation. The extent of mast cell degranulation was assessed both *in vitro*, by measuring release of β-hexosaminidase from cytoplasmic granules upon stimulation (18), and *in vivo*, by transferring mast cells into mast cell-deficient recipient mice (Kit$^{W\text{-}sh/W\text{-}sh}$) and performing passive cutaneous anaphylaxis (PCA) experiments. Even in the absence of p50, mast cells degranulated normally in response to IgE cross-linking or PMA and ionomycin stimulation, both *in vitro* and *in vivo* (Fig. 1D and E). In PCA experiments, efficient mast cell reconstitution of the ear pinna was confirmed by toluidine blue staining of paraffin-embedded tissue sections (Fig. 1F).

Next, we evaluated the ability of p50ko mast cells to produce cytokines, and we focused mostly on cytokines, such as IL-13, that are highly expressed by mast cells and have essential and nonredundant roles in allergy and asthmatic responses (10, 23, 38). Control and p50ko cells were stimulated by either LPS or IgE cross-linking, and expression of IL-6, TNF-α, and IL-13 was assessed by intracellular cytokine staining (Fig. 2A to C). Interestingly, mast cells lacking p50 showed reduced cytokine production in response to both LPS and IgE-Ag complexes, which was not due to altered expression of either of the surface receptors FcεRI and TLR4 (Fig. 2D). Reduced cytokine expression was particularly evident for IL-13, which is the cytokine necessary and sufficient for asthmatic responses in models of experimental asthma (10, 38) (Fig. 2B and C). Despite the increased number of mast cells in the tissues of p50ko animals, reduced IL-13 production from p50ko mast cells may therefore contribute to the asthma-resistant phenotype and overall lack of eosinophilia and Th2 responses observed in these mice. However, our data collected so far could not explain the increased mast cell numbers observed in the tissues of p50ko mice, which is what we set out to investigate next.

Increased survival in the absence of p50 correlates with increased expression of antiapoptotic genes. Given the increased number of tissue mast cells despite the normal proliferation capacity, we investigated whether p50ko mast cells were significantly affected with respect to their ability to survive upon IgE cross-linking and/or withdrawal of essential cytokines. The essential survival factors IL-3 and/or SCF were removed from the culture medium, with or without concomitant stimulation with IgE, which is known to provide a survival signal for mast cells, by inducing antiapoptotic factors (14, 39). Under all conditions tested, p50ko cells showed a consistently increased ability to survive compared to the controls (Fig. 3A to C). As expected, stimulation with IgE partially rescued control cells from apoptosis, but this effect was much more pronounced in the absence of p50 (Fig. 3C). To get an insight into the molecular mechanism that could explain such enhanced survival, we evaluated the expression of candidate pro- and antiapoptotic genes. Indeed, NF-κB binding sites have been identified in promoters and enhancers of a number of inducible genes involved in cell death, including Bcl-2, A1, and Bcl-X_L (14, 39). In particular, Bcl-2 is a known regulator of IL-3 withdrawal-dependent apoptosis, while A1 is a specific regulator of IgE-dependent survival in mast cells (14, 39). We therefore investigated whether these factors may be involved in regulating survival in the absence of p50. To this end, p50ko and control cells either were left resting or were stimulated with IgE cross-linking or LPS, after which expression of pro- and antiapoptotic gene candidates was assessed by qRT-PCR (Fig. 3D to G). Interestingly, both *bcl2* and *A1* were upregulated in cells lacking p50. Such upregulation was already evident at basal levels, but it became even more pronounced upon stimulation with IgE-DNP or LPS (Fig. 3D and E). Expression of another prosurvival factor, Bcl-X_L, was slightly diminished in p50ko cells (Fig. 3F), as was the expression of the proapoptotic gene *bax* (Fig. 3G). Since Bcl-2 and (especially) A1 were already shown to be involved in regulating mast cell survival, it is likely that the overall net increase of prosurvival factors is the basis of the observed enhanced survival of p50ko cells. Our data therefore show that p50ko mast cells exhibit increased survival in response to a variety of stimuli and that such enhanced survival is likely due to a profound alteration in the balance between pro- and antiapoptotic factors, with the latter being favored overall.

miR-146a regulates mast cell survival but not cytokine production or LPS desensitization. Since miRNAs are known to be involved in the regulation of a variety of cell functions, we assessed whether they are also involved in regulating the enhanced survival and reduced cytokine production observed in p50ko mast cells. We assessed in particular expression of two miRNAs (miR-146a and miR-221) that we found to be inducible in mast cells and to be dependent on NF-κB (18, 20, 36). p50ko and control mast cells either were left resting or were stimulated with PMA and ionomycin for 24 h, after which expression of miR-146a and miR-221 was assessed by qRT-PCR. miR-27a expression was also measured as a control, since it should not change upon stimulation of mast cells. Strikingly, p50ko mast cells specifically showed impaired expression of miR-146a in response to stimulation under all conditions tested, including PMA and ionomycin, IgE cross-linking, or LPS stimulation (Fig. 4A and B), indicating that p50 is indeed absolutely required for the expression of this miRNA. In contrast, levels of expression of miR-221, also known to be inducible upon mast cell stimulation in a NF-κB-dependent manner (20), were comparable between p50ko cells and controls, indicating that for miR-221 expression, but not miR-146a expression, the lack of p50 can be compensated for by the presence of other NF-κB subunits.

Since p50ko cells are completely unable to induce miR-146a expression and since it is known that miR-146a is itself a regulator of NF-κB activation (36), we asked whether at least part of the phenotype observed in mast cells in the absence of p50 could be due to the inability of these cells to induce miR-146a expression.

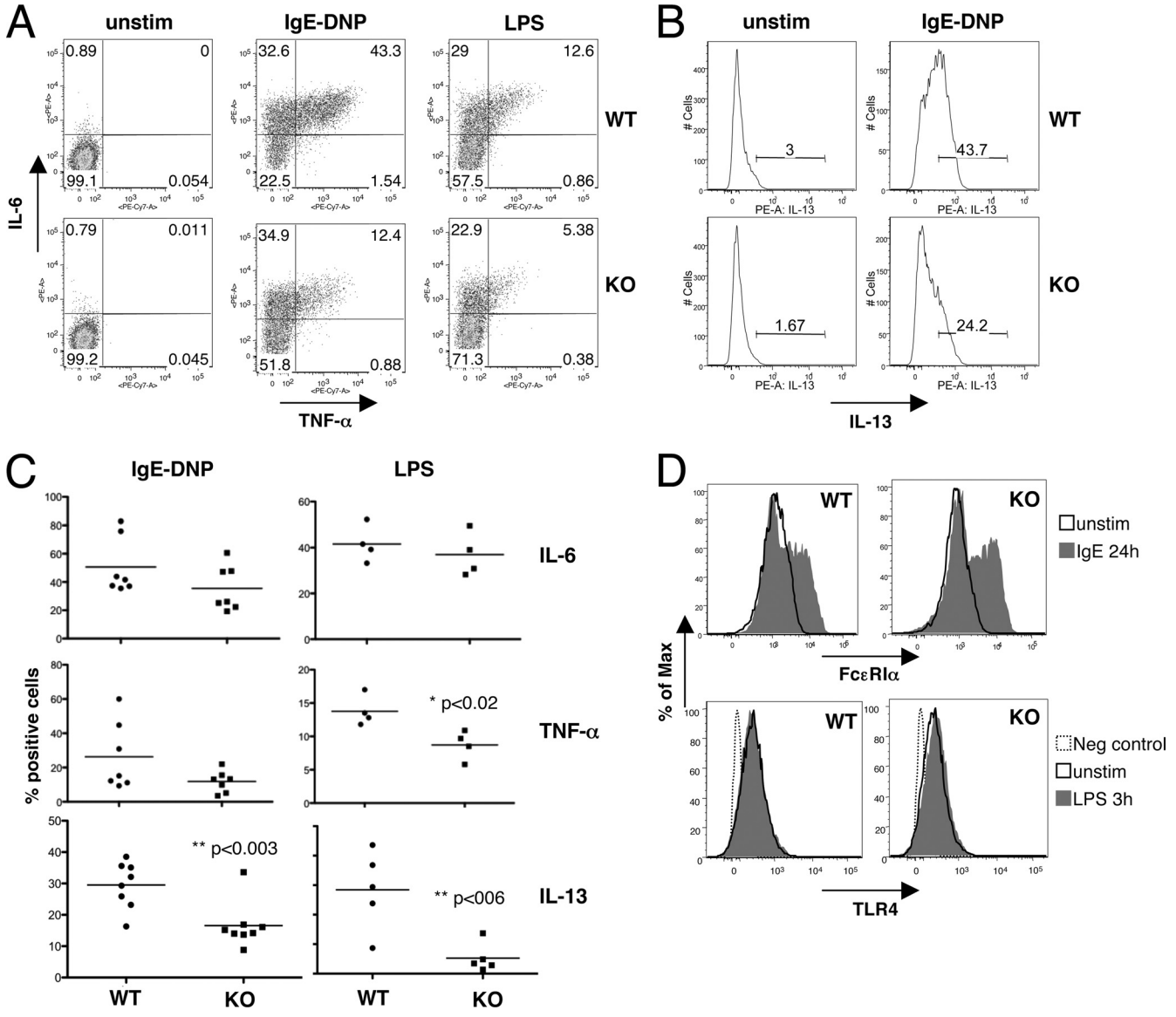

FIG 2 Reduced cytokine production in the absence of p50. (A) Cells either were left untreated or were stimulated with IgE-Ag complexes or LPS prior analysis of IL-6 and TNF-α expression by intracellular cytokine staining. (B) Same as described for panel A except that IL-13 production is shown. (C) Same as described for panel A except that the percentages of cells positive for the indicated cytokine are provided. Each dot represents data from one independent experiment. (D) (Top) Surface expression of FcεRIα in control and p50ko mast cells left unstimulated or stimulated with IgE and antigen for 24 h to assess cell ability to upregulate FcεRIα expression. (Bottom) Expression of TLR4 on the surface of control and p50ko mast cells left unstimulated or stimulated with LPS for 3 h.

We therefore transduced mast cells with either a control lentiviral vector (shLuc, expressing an irrelevant hairpin) or a vector expressing miR-146a, and we asked whether miR-146a expression led to altered survival in mast cells. Indeed, mast cells transduced with miR-146a showed consistently increased cell death compared to control cells, even when cultured in medium supplemented with all survival factors (Fig. 4C and D). As a control, to confirm that our lentivirally derived miR-146a was indeed properly functional, we evaluated levels of expression of the known miR-146a target Traf6 (36). Western blot analysis showed a significant decrease in Traf6 expression in both p50ko and control cells whenever miR-146a was expressed (Fig. 4E). Interestingly, forced miR-146a expression led to an only modest reduction of *bcl2* expression in p50ko cells, indicating that although miR-146a works in the same pathway as p50, forced miR-146a expression is not sufficient to completely compensate for and restore the phenotype induced by the lack of p50 (Fig. 4F). Along the same lines, we did not observe any particular effect of miR-146a on IL-6 and TNF-α expression (Fig. 4G).

Repeated stimulation of cells can result in loss of responses. miR-146a is known to regulate tolerance of LPS in macrophages (36), and, given the inability of p50ko mast cells to induce miR-146a expression in response to LPS, we assessed whether these cells might be impaired in their responses to sequential stimulations with LPS. However, mast cells lacking p50 showed a reduction in cytokine production in response to acute LPS stimulation

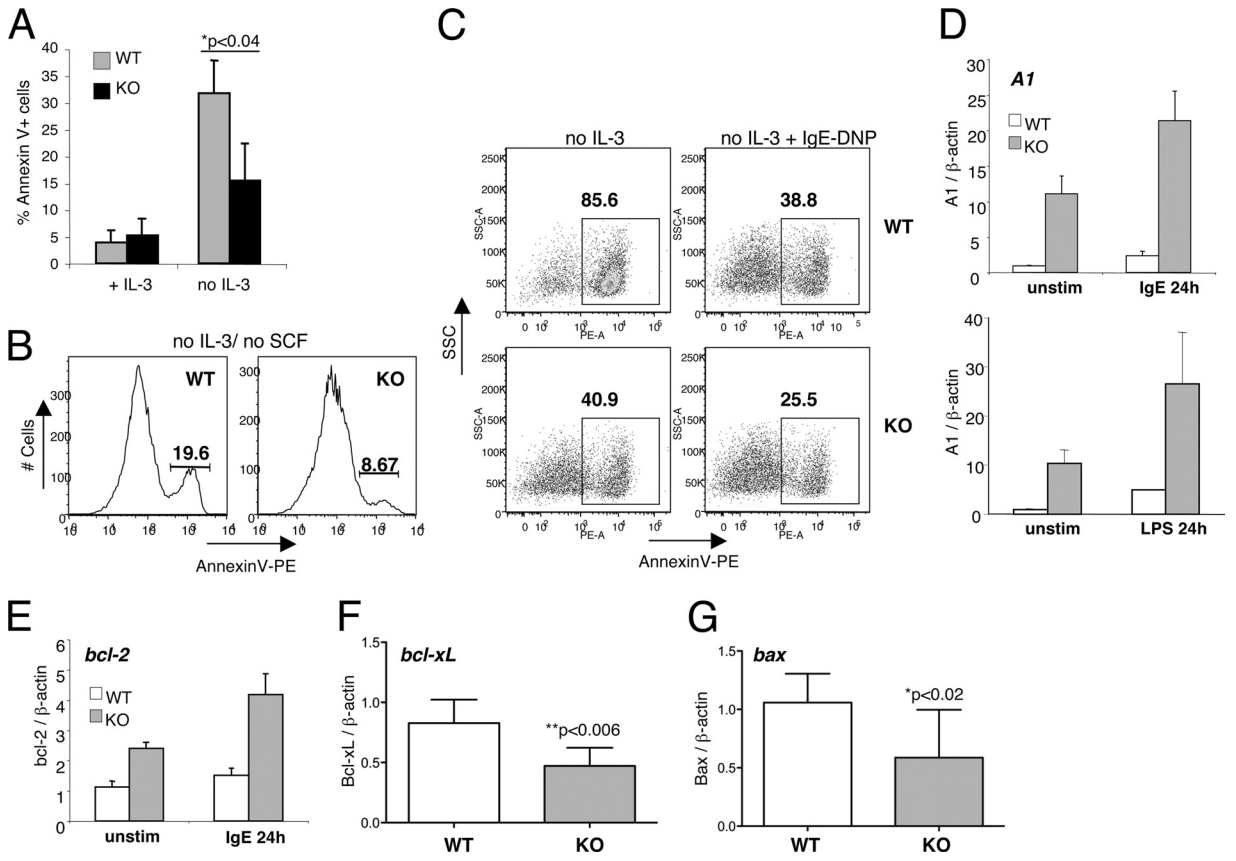

FIG 3 Enhanced survival and increased expression of antiapoptotic factors in the absence of p50. (A and B) Mast cells were cultured with IL-3 alone (A) or IL-3 plus SCF (B) prior removal of IL-3 or SCF for ~4 days. Analysis of cell death was performed by annexin V staining. The data in the graph in panel A represent the means of the results of four independent experiments, while panel B is representative of the results of at least two experiments. (C) Same as described for panel A except that cells either were left unstimulated or were stimulated with IgE and antigen at the time of initial IL-3 withdrawal. Data represent the results of one of two experiments. (D) p50ko and control cells either were left resting or were stimulated for 24 h with IgE-Ag complexes (top) or LPS (bottom). Total RNA was extracted, and *A1* mRNA expression was assessed by qRT-PCR. Data represent the results of one of four experiments. (E) Same as described for panel D except that expression of *bcl2* was assessed. Data represent the results of one of four experiments. (F and G) Expression levels of *bcl-X*$_L$ (F) and *bax* (G) were analyzed by qRT-PCR in unstimulated control and p50-deleted mast cells. Data represent the results of one experiment of four for *bax* and of three for *bcl-X*$_L$.

but then responded similarly to control cells, becoming unresponsive to subsequent stimulations with LPS (see Fig. S1B to C in the supplemental material), indicating that such LPS-dependent unresponsiveness in mast cells is not strictly dependent on the presence of miR-146a.

miR-146a is highly expressed in the T cell memory compartment from both human and mouse. Since we previously reported that miR-146a is differentially expressed by Th1 and Th2 T cell subsets in the mouse (22), we asked whether the novel molecular network we identified in mast cells, involving miR-146a and p50, could also be at play in regulating T lymphocyte polarization, which was also shown to be altered in mice in the absence of p50 (5). In mouse CD4 T cells, we found that miR-146a was indeed expressed at higher levels in Th1 cells than in Th2 cells (Fig. 5A); however, it remained inducible in both T cell subsets upon restimulation, with strong T cell receptor (TCR) stimulation correlating with high levels of miR-146a expression and also with high and sustained numbers of cell cycles, as assessed by CFSE dilution (Fig. 5B). We therefore hypothesized that miR-146a could have a role in regulating T cell activation and expansion rather than T cell polarization. In line with this hypothesis, we found that miR-146a expression was consistently elevated in the effector and memory compartments (T_{EM} and T_{CM}) in both CD4 and CD8 murine T cells (Fig. 5C and D). Similarly to CD4 T cells, naïve CD8 T cells activated *in vitro* with anti-CD3 and anti-CD28 showed increased expression of miR-146a (Fig. 5D, left panel). Next, we reasoned that if miR-146a is an important regulator of T cell polarization and/or activation, such a process should be conserved also in human T cells. Human Th1 and Th2 cells, either differentiated *in vitro* or separated *ex vivo* from peripheral blood, did not show any differential expression of this miRNA (Fig. 5E; see also Fig. S2A in the supplemental material). However, similarly to mouse T cells, resting human CD4 lymphoctyes retained the ability to activate expression of miR-146a once restimulated and, even more importantly, miR-146a was expressed at high levels in memory T cells isolated *ex vivo* from peripheral blood (Fig. 5F and G). These data indicate that miR-146a is unlikely to play a major role in T cell polarization in either human or mouse except (probably) in Treg cells, in which it is expressed at high levels (17; see also Fig. S2A in the supplemental material), but point toward a role in regulating lymphocyte activation and/or in the establishment of immunological memory.

miR-146a influences cell expansion but not cell death of primary human lymphocytes. To further investigate the role of miR-

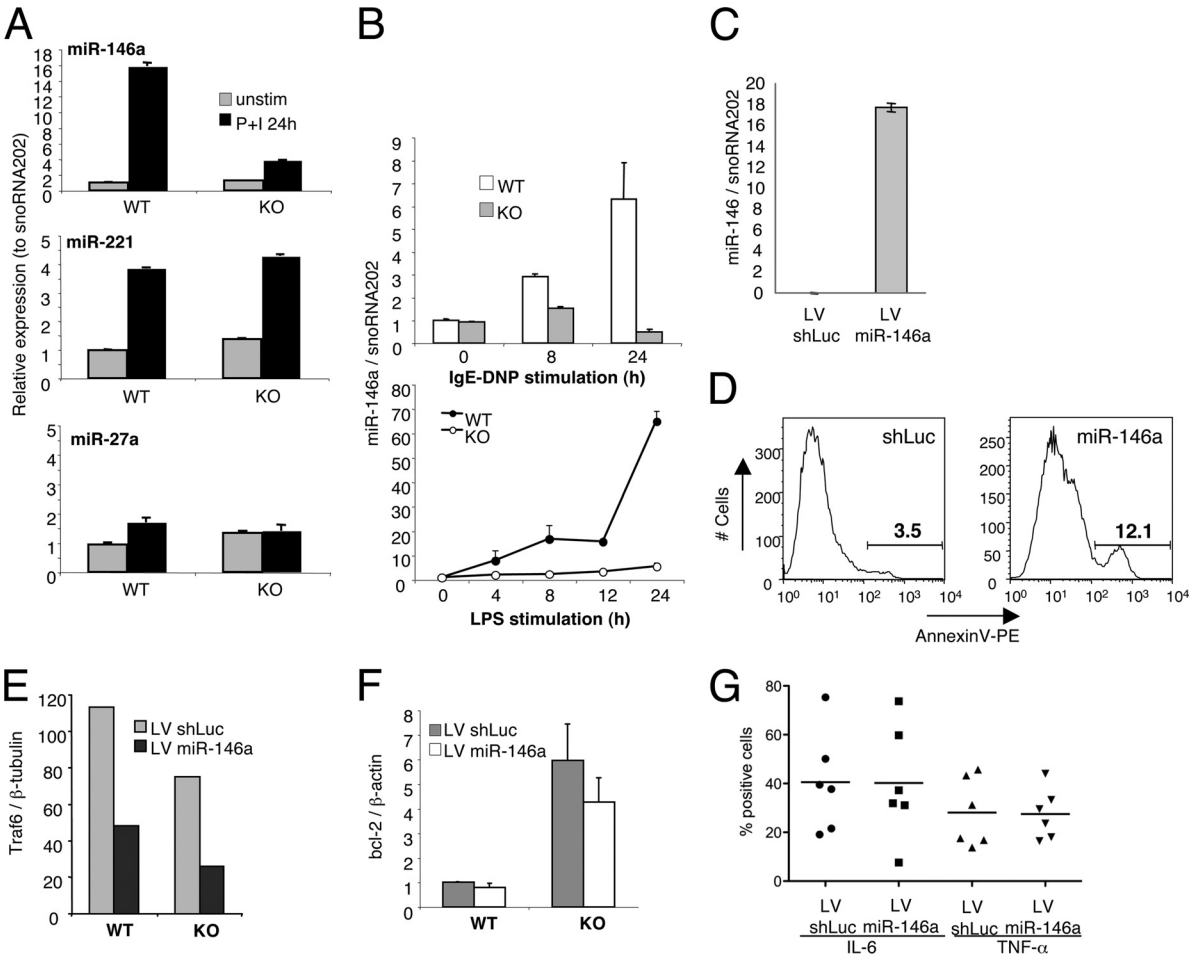

FIG 4 miR-146a is not expressed in mast cells in the absence of p50. (A) Differentiated mast cells either were left resting or were stimulated with PMA and ionomycin (P+I) for 24 h prior analysis of the expression of miR-146a, miR-221, and miR27a by qRT-PCR. snoRNA202 was used as an endogenous control. Data represent the results of one of three experiments. (B) Same as described for panel A except that cells were stimulated with either IgE and antigen (top) or LPS (bottom) for the indicated times. Data represent the results of one of three experiments. (C) Mast cells were lentivirally (LV) transduced to express either miR-146a or an irrelevant hairpin as a control (shLuc). miR-146a expression was assessed by qRT-PCR. (D) Cells treated as described for panel C were analyzed for apoptosis by annexin V staining. (E) Cells treated as described for panel C were lysed in Laemmli sample buffer, and expression of Traf6 was analyzed by Western blotting. β-Tubulin was used as loading control, and quantification was performed using an image reader. Data represent ratios between the Traf6 and β-tubulin signals determined in one of two independent experiments with similar results. (F) Same as described for panel C except that levels of expression of *bcl2* were analyzed by qRT-PCR. (G) Cells lentivirally transduced as described for panel C were stimulated with IgE and antigen prior intracellular cytokine staining to assess IL-6 and TNF-α expression. Each dot represents results of one independent experiment.

146a specifically in human lymphocytes, and to assess whether it could modulate cell survival, similarly to mast cells, human CD4 T cells were lentivirally transduced to express GFP, alone or in combination with miR-146a (Fig. 6A). Levels of miR-146a in transduced cells were overall lower than or comparable to the physiological levels of endogenous expression observed in lymphocytes upon TCR stimulation (see Fig. S2B in the supplemental material). However, we found that primary human T cells expressing miR-146a did not show any significant alteration in Fas-mediated cell death or in CD95 expression or in the ability to survive in response to withdrawal of IL-2 (see Fig. S2C to D in the supplemental material; also data not shown). Moreover, we observed no significant effect of miR-146a on IL-2, IFN-γ, or IL-4 cytokine production (see Fig. S2E in the supplemental material; also data not shown).

While we did not observe any effect on cell death, memory human T cells expressing miR-146a showed increased expansion after restimulation with anti-CD3 and anti-CD28 (Fig. 6B). Specifically, cells expanded similarly up to ~day 4 to 5 after stimulation, regardless of the absence or presence of miR-146a expression, but miR-146a-expressing cells continued to expand more vigorously than control cells in the subsequent days, an effect that was especially evident in the absence of exogenous IL-2, indicating that this miRNA could act directly in response to TCR stimulation to favor cell expansion (Fig. 6B). The fact that cytokine production was largely unaffected by miR-146a under all conditions tested ruled out the possibility that miR-146a-expressing cells could sustain their own proliferation through increased IL-2 production. We also performed adoptive transfer experiments in which mouse OT-II naïve T cells transfected with either a miR-146a mimic or a nontargeting control were transferred into a recipient mouse. A few days after challenge with OVA, both the draining lymph node and the spleen showed a slightly higher percentage of transferred cells whenever miR-146a was present, sug-

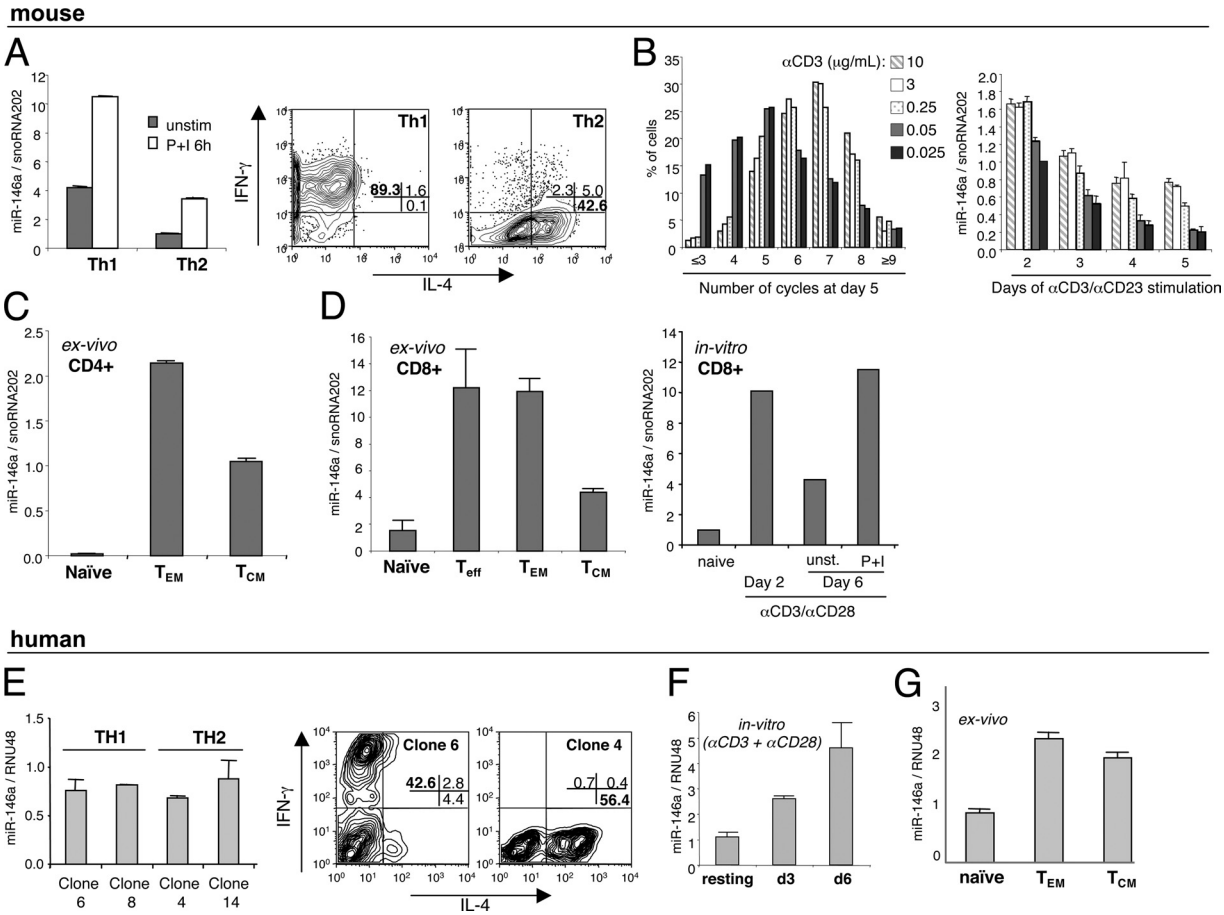

FIG 5 Memory T cells express high levels of miR-146a expression, which is induced by TCR stimulation. (A) Naïve CD4 T cells were isolated from the spleen and lymph nodes of C57BL/6 or OT-II mice and differentiated to either Th1 or Th2. At day 5, cells either were left resting or were restimulated with PMA and ionomycin for 6 h prior RNA extraction and qRT-PCR for miR-146a (left) and intracellular cytokine staining to verify polarization (right). snoRNA202 was used as an endogenous control. (B) Naïve CD4 T cells were isolated from the spleen and lymph nodes of C57BL/6 or OT-II mice, labeled with CFSE, and stimulated with the indicated concentrations of anti-CD3 in the presence of anti-CD28 for 48 h. The numbers of cycles were counted based on CFSE dilution (left), and miR-146a expression was evaluated in the same samples by qRT-PCR (right). (C) Naïve CD4 T cells were isolated from the spleen and lymph nodes of OT-II mice and were transferred i.v. into recipient C57BL/6 mice prior challenge with OVA. At 5 and 15 days after transfer, T_{EM} and T_{CM} were subjected to FACS analysis (for T_{EM}, $CD62L^{lo}$ $CD44^{hi}$ $CD127^{hi}$; for T_{CM}, $CD62L^{hi}$ $CD44^{hi}$ $CD127^{hi}$) and miR-146a expression was measured by qRT-PCR. (D) (Left) Same as described for panel C except that OT-I naïve T cells were used and mice were challenged with either OVA or SIINFKL peptide. (Right) Purified naïve CD8 cells were stimulated *in vitro* with plate-bound anti-CD3 and anti-CD28 for 2 days and further expanded up to day 6 with rIL-2 (10 to 100 U/ml), after which they either were left resting or were restimulated for 6 h with PMA and ionomycin (P+I) prior analysis of miR-146a expression. (E) miR-146a expression in human Th1 and Th2 clones was assessed by qRT-PCR (left). RNU48 was used as an endogenous control. Clones were characterized by the expression of surface markers and by the expression of IFN-γ and IL-4 (right). (F) Resting primary human CD4 cells either were immediately lysed in TRIzol or were stimulated with plate-bound anti-CD3 and anti-CD28 for 3 and 6 days prior analysis of miR-146a expression by qRT-PCR. (G) Primary human CD4 T cells were sorted from peripheral blood as follows: for naïve cells, $CD4^+$ $CD8^-$ $CD25^-$ $CD45RA^+$ $CCR7^+$; for T_{EM} cells, $CD4^+$ $CD8^-$ $CD25^-$ $CD45RA^-$ $CCR7^-$; and for T_{CM} cells, $CD4^+$ $CD8^-$ $CD25^-CD45RA^-$ $CCR7^+$. Cells were lysed in TRIzol immediately after sorting, and miR-146a expression was assessed by qRT-PCR.

gesting increased expansion abilities following challenge (see Fig. S3A and B in the supplemental material). Finally, in a different experimental setting, human naïve T cells were transiently transfected with a miR-146a mimic and then stimulated with allogeneic peripheral blood mononuclear cells (PBMC), after which the percentage of activated T cells was assessed. Consistently with the results obtained using lentiviral vectors, we observed a reproducible, albeit small, increase in the percentage of activated cells whenever miR-146a was present (see Fig. S3C in the supplemental material).

miR-146a regulates CCR7 expression. While IL-2 does not significantly contribute to the initial cycling of antigen-stimulated T cells, it is nevertheless necessary for the successful generation of memory responses (12). Indeed, the IL-2 signal strength has been shown to contribute to the differentiation of murine CD8 T_{EM} and T_{CM} (28). In our culture system, the absence of IL-2 uncovered a T_{EM}-like phenotype, with an inability to sustain CCR7 expression and expansion after TCR stimulation (Fig. 6C and D). Interestingly, miR-146a expression determined a reduction of CCR7 expression under all conditions tested, indicating not only that miR-146a contributed to T cell expansion upon TCR stimulation but also that it may modulate the establishment of a T_{EM}-like phenotype (with reduced CCR7 expression) in primary human lymphocytes (Fig. 6E). Since most of our experiments thus far were performed on memory Th1, Th2, and Th17 cell subsets isolated from peripheral blood and restimulated *in vitro*, to better assess the

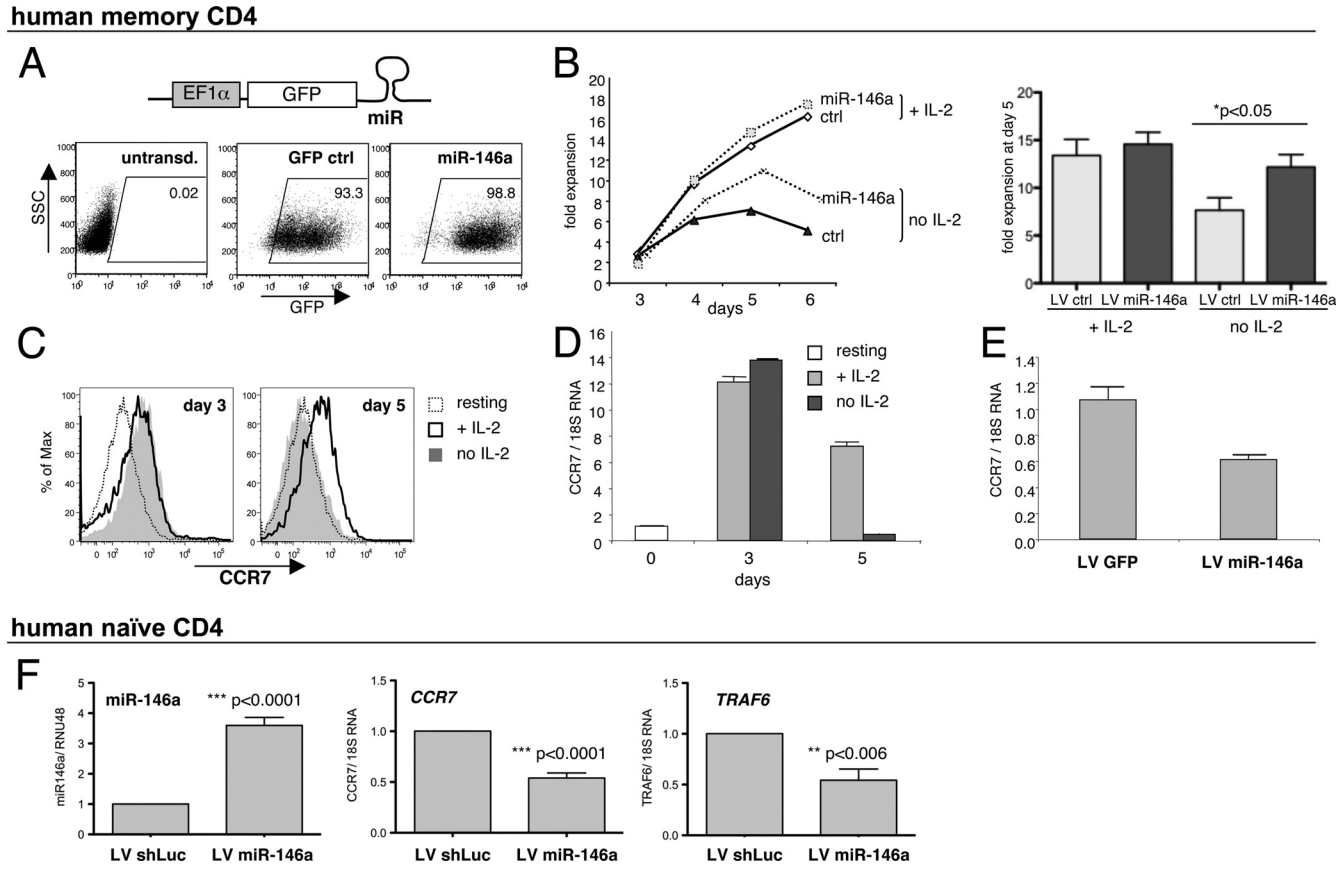

FIG 6 miR-146a expression in T cells led to enhanced expansion and reduced CCR7 expression upon TCR stimulation. (A) (Top) Schematic of the lentiviral vector used. The control vectors expressed GFP alone or in combination with a nontargeting hairpin or an shRNA against luciferase. (Bottom) Primary human T cells ($CD4^+$ $CD45RA^-$ $CD25^-$ $CD8^-$) transduced with the indicated vector were sorted for GFP expression 2 to 5 days after transduction. Data represent cells obtained after sorting in one representative experiment. After the initial experiments, Th1 ($CXCR3^+$), Th2 ($CCR4^+$), or Th17 ($CCR6^+$ $CCR4^+$) subsets were used interchangeably since they provided identical results. untransd., untransduced; ctrl, control. (B) Primary human T cells transduced with either a miR-146a- or control-expressing vector were stimulated for 48 h on plate-bound anti-CD3 and anti-CD28 in the presence or absence of IL-2 at 500 U/ml and then expanded for 6 days with or without addition of exogenous IL-2. Cell numbers were assessed daily and are plotted as fold expansion. The left panel shows data from one representative experiment, while the right panel shows data representing the mean results of four experiments (fold expansion at day 5 only). (C) CCR7 surface expression was assessed on cells treated as described for panel B. Stimulated cells were homogenously $CD45RA^-$ $CD25^+$. Max, maximum. (D) *CCR7* mRNA expression was assessed by qRT-PCR in cells as described for panel C. (E) Primary human T cells were transduced with the indicated lentiviral vectors, and *CCR7* mRNA expression was assessed 3 to 5 days after transduction. Data are representative of the results of 4 independent experiments performed under various conditions (i.e., with or without exogenous IL-2, at resting state, or upon restimulation with anti-CD3 and anti-CD28 for 3 days), all with comparable results. (F) Primary naïve human T cells transduced with the indicated vectors were stimulated with plate-bound anti-CD3 and anti-CD28 for 5 days prior qRT-PCR to determine expression of miR-146a, *CCR7*, and *TRAF6*. Data are representative of the results of one experiment of two.

effect of miR-146a during the first antigenic stimulation of naïve T cells, we lentivirally transduced human naïve T cells freshly isolated from peripheral blood to express miR-146a. Three days after transduction, GFP^+ cells were sorted and stimulated with plate-bound anti-CD3 and anti-CD28, and, 5 days after stimulation, total RNA was extracted and levels of expression of miR-146a and *CCR7* were assessed by qRT-PCR. As a control, we also measured expression of the known miR-146a target *TRAF6*. Similarly to memory cells, naïve T cells expressing miR-146a showed reduced *CCR7* and *TRAF6* expression (Fig. 6F). However, the reduction in CCR7 expression was very selective, as we could not observe any alteration in the expression levels of CD25, CD45RA, CD45RO, CD62L, and CD127 (data not shown). Moreover, such an effect of miR-146a is unlikely to be exerted directly on CCR7, which is not a predicted target for this miRNA (16).

It was previously shown that mice with a T cell-specific deletion of TRAF6 mounted robust CD8 effector responses but had a profound defect in their ability to generate memory cells (26). To further demonstrate that TRAF6 is indeed a target for miR-146a in primary T lymphocytes, we also performed reporter assay experiments using the 3′ untranslated region (3′UTR) of *TRAF6* and found that it was efficiently targeted by miR-146a in both Jurkat cells and primary murine Th2 cells (see Fig. S3D in the supplemental material). Our data point toward a role for miR-146a in regulating human T cell responses and memory formation, possibly through the modulation of TRAF6 expression.

Dysregulated T cell memory formation in the absence of the p50/miR-146a molecular circuitry. Since we showed that miR-146a dysregulation could influence T cell memory formation, leading to a $CCR7^{lo}$, T_{EM}-like phenotype, and we also found that in mast cells miR-146a expression was fully dependent on p50 expression, we looked into T cell activation and memory forma-

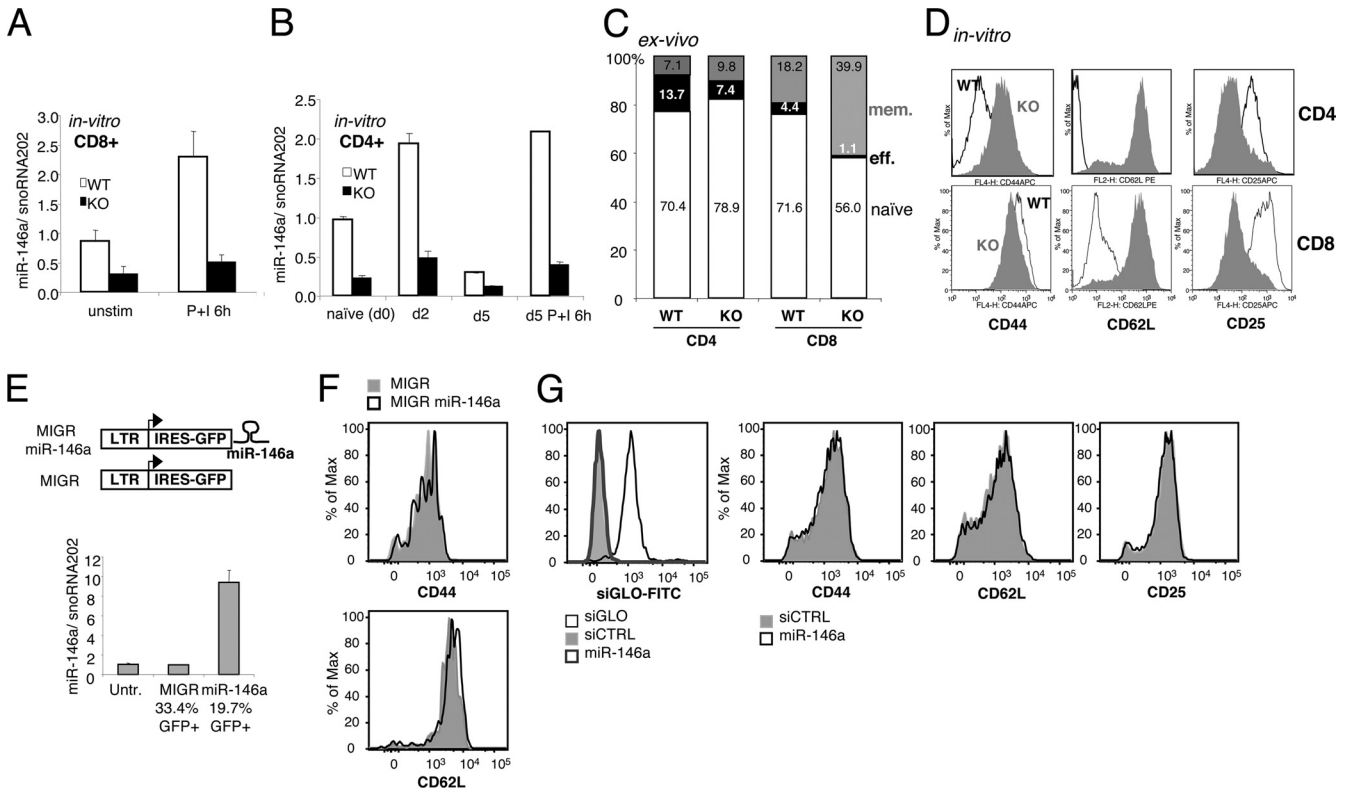

FIG 7 T_{CM} differentiation in the absence of p50 and miR-146a. (A and B) $CD8^+$ (A) or $CD4^+$ (B) naïve T cells were sorted from the spleen and lymph nodes of p50ko and control mice at day 0 (d0) and either were immediately lysed in TRIzol or were stimulated for 2 to 5 days with plate-bound anti-CD3 and anti-CD28, after which they either were left resting or were restimulated with PMA and ionomycin (P+I) for 6 h. RNA was extracted and expression of miR-146a assessed by qRT-PCR. (C) Spleen and lymph nodes were collected from p50ko and control mice (3 mice per group), and the percentages of naïve ($CD62L^+$ $CD44^-$), effector ($CD62L^-$ $CD44^+$) (eff.), and memory ($CD62L^+$ $CD44^+$) (mem.) cells were evaluated in the CD4 and CD8 compartments. (D) Naïve CD4 and CD8 T cells from the spleen and lymph nodes of p50ko and control mice (3 mice per group) were subjected to FACS analysis and were stimulated for 5 days with plate-bound anti-CD3 and anti-CD28, after which expression of CD44, CD62L, and CD25 was assessed by FACS staining. (E) (Top) Schematic representation of the retroviral vector used for transduction of primary murine T cells, expressing either GFP alone or GFP and miR-146a. Expression of vector-derived miR-146a was evaluated in separate transduction experiments using total wild-type $CD4^+$ T cells (qRT-PCR graph at the bottom). Indicated below the bars are the percentages of GFP^+, transduced cells. Untr., untransduced control. (F) Sorted naïve $CD4^+$ T cells ($CD62L^{hi}$ $CD44^{lo}$) from p50ko mice were transduced with the indicated retroviruses 48 h after initial activation with plate-bound anti-CD3 and anti-CD28, and expression of CD62L and CD44 was assessed at day 5 after stimulation (day 3 after transduction). Cells shown in the histograms were gated on the GFP^+ cells. (G) To assess whether changes in miR-146a expression during the first 2 days of T cell stimulation could be essential for the final outcome on the phenotype, which we could not investigate by using retroviruses, sorted naïve p50ko $CD4^+$ T cells were transiently transfected with Amaxa prior anti-CD3/anti-CD28 stimulation. Transfection efficiency was assessed by using a nontargeting fluorescent oligonucleotide (siGLO; left panel). Expression of CD44, CD62L, and CD25 was assessed at days 2, 3, and 4 after transfection. Data represent the day 3 results of one experiment of two.

tion in the absence of p50 (and therefore in the absence also of miR-146a). First, we assessed whether miR-146a expression was indeed impaired in the absence of p50 also in T lymphocytes. Naïve CD8 and CD4 T cells were isolated from p50-deleted animals and were stimulated *in vitro* with plate-bound anti-CD3 and anti-CD28 for 5 days, after which they either were left resting or were restimulated with PMA and ionomycin (P+I) for 6 h. Similarly to mast cells stimulated with either IgE or LPS, T cells (both CD4 and CD8) lacking p50 were completely unable to induce miR-146a expression either upon TCR engagement of naïve T cells or upon restimulation (Fig. 7A and B). Moreover, there was no significant difference between p50-deleted T cells and controls in IFN-γ, IL-4, and IL-2 cytokine production, as assessed by intracellular cytokine staining (data not shown). We therefore analyzed the memory compartment of p50-deleted mice *ex vivo*. As shown in Fig. 7C, in the absence of p50, both the CD4 and (especially) the CD8 T cell compartments showed a striking increase in the memory compartment, and a reduction of effector cells, even in the absence of any challenge. Importantly, sorted p50ko naïve T cells differentiated *in vitro* for 5 days also showed a T_{CM}-like phenotype, with high expression of CD44 and CD62L and intermediate expression of CD25 (Fig. 7D), indicating that the absence of p50 and miR-146a intrinsically favors naïve T cell differentiation to a T_{CM} phenotype, independently of thymic development. However, forced expression of miR-146a in p50ko CD4 T cells by either retroviral transduction or transient transfection could not rescue the phenotype observed in the absence of p50 (Fig. 7E-G), indicating once again that the presence of miR-146a alone was not able to fully compensate for the lack of a transcription factor, at least in our *in vitro* experimental settings.

DISCUSSION

NF-κB is involved in regulating many aspects of cellular activity during an immune response, and activation of this transcription

factor by receptors of the innate and adaptive immune response is essential for host defense (reviewed in reference 25). Almost all danger-sensing receptors activate NF-κB to mediate effector functions; however, NF-κB-driven responses must be promptly terminated once danger is eliminated, as aberrant NF-κB activity can directly lead to uncontrolled tissue damage and disease (reviewed in reference 30).

Here, we found that the absence of one specific NF-κB family member, p50, improved mast cell survival in response to a variety of signals. The underlying mechanism for such enhanced survival involved increased expression of prosurvival factors such as Bcl-2 and A1, as well as reduced expression of proapoptotic factors such as Bax and miR-146a, which in this context acted as a proapoptotic factor and as a modulator of NF-κB signaling by targeting Traf6. In our current working model, NF-κB activation in mast cells can occur as a result of FcεRI cross-linking or TLR4 engagement, leading to nuclear translocation of p65-p50 heterodimers that activate transcription of both antiapoptotic factor genes such as *bcl2* and *A1*) as well as of *pri-miR-146a*, which acts as a negative regulator of NF-κB activation (see Fig. S4A to C in the supplemental material). In the absence of p50, not only are some survival factors strongly upregulated, but the negative feedback on NF-κB activation is lost, as miR-146a cannot be expressed, reinforcing the positive survival signal. The fact that in the context of mast cell survival miR-146a acts as a proapoptotic factor is also highlighted by the fact that forced expression of miR-146a leads to increased cell death.

What remains to be investigated is the mechanism by which NF-κB p50 may act as a positive regulator of some genes (namely, miR-146a, for which p50 is essential) and as a negative regulator of other genes (namely, Bcl-2 and A1, whose expression is increased under both basal and activated conditions in the absence of p50). One possible explanation concerns the formation of compensatory heterodimers in the absence of p50 with an altered pattern of binding and activation. Indeed, the unique DNA-binding properties of distinct NF-κB dimers influence the selective regulation of NF-κB target genes. NF-κB dimers can be separated in three distinct DNA-binding classes based on their DNA-binding preferences: p50 or p52 homodimers, heterodimers, and c-Rel or RelA homodimers (35). Clearly, sites that are normally preferentially bound by p50 homodimers could become "free" to be bound by other NF-κB family members in the absence of p50, altering regulation of transcription at these specific sites. Our initial data in this direction show that p65 nuclear translocation is normal in mast cells in the absence of p50; however, its overall expression is slightly reduced (see Fig. S1D in the supplemental material). We have yet to uncover specific differences in promoter binding, which is to be the topic of future work. Another possibility is that, as a consequence of lacking a transactivating domain, p50 homodimers negatively regulate expression of *bcl2* and *A1* under resting conditions and that the negative regulation is overcome by other activating heterodimers upon activation. For example, negative regulation by p50 homodimers has been reported to correlate with repression of NF-κB-driven transcription in tolerant T cells (9), and tolerance of LPS in monocytes has been shown to involve LPS-dependent mobilization of NF-κB with a predominance of p50 homodimers (43). p50 homodimers are also known to be nuclear even in the absence of stimulation, which may explain the increased expression of Bcl-2 and A1 at basal levels in the absence of p50 (2, 32). On the other hand, miR-146a expression remained exquisitely dependent on the presence of p50 under all conditions tested. Further experiments are planned to elucidate the exact molecular mechanism responsible for the enhanced survival observed in mast cells in the absence of p50.

As for the observed increase of mast cells in the tissues of p50ko animals, the possibility remains that such alteration arises from an increased ability of hematopoietic stem cells (HSC) in the bone marrow to differentiate more promptly to mast cells and/or in general to the myeloid lineage. The increased number of mast cells could therefore be a composite effect of increased differentiation from stem cells combined with a cell-intrinsic ability of the cells to survive in response to a variety of stimuli. A detailed analysis of HSC differentiation in the absence of p50 would surely provide new insights into the role of this transcription factor in hematopoietic differentiation, particularly to the myeloid lineage.

In contrast to mast cell results, in T lymphocytes miR-146a did not regulate cell death but rather influenced T cell activation upon TCR engagement. It is interesting that, in accordance with published data (4), we found that ectopic expression of miR-146a in Jurkat cells led to a mild reduction in apoptosis (data not shown). However, primary cells expressing miR-146a did not show any significant alteration in Fas-mediated cell death or in CD95 expression. Such a discrepancy may be due to the fact that, whereas for Jurkat cells, activation-induced cell death (AICD) can be completely cell autonomous, primary T lymphocytes undergo AICD by making contact with their activated neighbors, resulting in "fratricide" rather than "suicide" (3, 6, 7). Alternatively, miR-146a may have roles that vary in the different cell types depending on the type and relative abundance of mRNA targets that constitute the transcriptome of that specific cell, as we have shown for mast cells and T lymphocytes. We therefore propose a model in which miR-146a upregulation upon TCR stimulation contributes to the overall strength of signal arising from the TCR, by favoring cell activation and cell expansion, and by modulating the establishment of immunological memory, in particular, by favoring a $CCR7^{lo}$, T_{EM}-like phenotype (see Fig. S4D in the supplemental material).

Importantly, it has recently been shown that mice lacking p50 have altered negative selection in the thymus and develop a population of single-positive CD8 thymocytes with memory T cell-like properties that populate peripheral immune organs (11). Here, we were able to show that in the absence of p50, even highly purified naïve CD4 and CD8 T cells stimulated *in vitro* preferentially acquired a T_{CM}-like phenotype, with high expression of CD62L and moderate expression of CD25. While this effect was clearly dependent on the absence of p50, we speculate that it might also have been due, at least in part, to the inability of the cells to express miR-146a in response to TCR stimulation. It would be interesting to estimate the proportion of the effect of a given transcription factor, such as NF-κB p50, that goes through the altered expression of a specific miRNA, such as miR-146a. However, it has to be noted that miR-146a-deleted mice showed normal proportions of CD4 and CD8 cells both in the thymus and in the periphery (17), and even in our hands in mast cells, miR-146a was only partially able to compensate for the lack of p50, indicating that at least in this case, the effect of a transcription factor remains predominant compared to that of the miRNA. Moreover, some of our data are also in line with a very recent publication showing that miR-146a also controls the resolution of T cell responses in

mice and that its absence leads to increased survival through modulation of *bcl2* expression (40).

As for targets of miR-146a, we found that TRAF6 was clearly targeted by this miRNA both in mast cells and primary T lymphocytes from human and mouse, similarly to what has been extensively shown for macrophages and other cell types (36). However, other targets for miR-146a have been suggested (17, 31), and TRAF6 may not be the only or the most relevant target for this miRNA in these particular cell contexts: for example, we showed for miR-221 that, although some specific genes are targeted by this miRNA in mast cells, the effect of miR-221 does not predominantly operate through these targets, and bioinformatics analysis clearly showed that miR-221 affected a few hundred primary and secondary targets (18). Nevertheless, in an attempt to phenocopy the effects of miR-146a, we attempted TRAF6 knockdown experiments in primary human T cells using small interfering RNAs (siRNAs). While, unfortunately, we have so far been unable to draw definite conclusions from these experiments, it remains to be noted that mice with a T cell-specific deletion of *traf6* mounted robust CD8 effector responses but had a profound defect in their ability to generate memory cells (26), a result which was therefore reminiscent of the increased T_{EM}-like phenotype of our miR-146a-expressing cells. Moreover, Fas-mediated apoptosis in the absence of Traf6 was normal, and cells showed increased proliferation in response to TCR stimulation, again similarly to what we observed in miR-146a-expressing cells (15).

Finally, as for the fact that miR-146a is expressed at a higher level in Th1 murine lymphocytes than in Th2 (22), we think that such differential expression might be related to the strength of signal that favors one or the other phenotype, with stronger stimulation favoring Th1 responses (37). Further supporting this possibility, it has been suggested that strong stimulation is needed for commitment to T_{EM} cells, whereas weaker stimulation favors the generation of less-committed T_{CM} cells (27), indicating that miR-146a might play an important role in lowering the strength of signal required for full T cell activation and therefore mimic stronger TCR engagement, resulting in increased activation and generation of T_{EM}. Alternatively, IL-4 and/or IL-12 signaling may also contribute to modulate expression of miR-146a in conjunction with TCR stimulation, a possibility that remains to be investigated. However, it has to be noted that we found no significant differences in the levels of miR-146a expression in human Th1, Th2, and Th17 cells, indicating that cytokine signaling may not be at play (see Fig. S2A in the supplemental material). Similarly to the mouse results (17), this miRNA was instead expressed at very high levels in human Tregs.

Overall, our findings indicate that NF-κB p50 acts in a cell-autonomous manner in differentiated mast cells to favor survival in response to withdrawal of essential cytokines or antigenic stimulation, while in T lymphocytes it enhances TCR-dependent activation and modulates memory formation. This novel molecular network comprising specifically p50 and miR-146a and regulating cell survival, tissue homeostasis, and T cell activation may have important implications for our understanding of the physiologic responses occurring, for example, during infections with helminth parasites or allergic reactions and possibly even in mast cell disorders such as systemic mastocytosis.

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We have no conflict of interest to declare.

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